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FULL PAPER

Cloning and expression analysis of putative glyceraldehyde-3-phosphate dehydrogenase genes in *Pilobolus crystallinus*

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Abstract Putative glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) genes were cloned from *Pilobolus crystallinus* with degenerated primers designed from conserved sequences in many GAPDHs. *P. crystallinus* had three *gapdh* homologue genes, named *pcgapdh1*, *pcgapdh2*, and *pcgapdh3*. Deduced amino acid sequences for PCGAPDH1, PCGAPDH2, and PCGAPDH3 showed highest similarity with GPD3, GPD1, and GPD2, respectively, of *Mucor circinelloides*, indicating that these three *gapdh* genes had diverged before *Pilobolus* and *Mucor* were separated. The expression patterns of the *gapdh* genes, however, were quite different between *P. crystallinus* and *M. circinelloides*. All the three *pcgapdh* genes were expressed, and the expression of *pcgapdh2* was suppressed by glucose and sodium acetate. These results indicate that the function of these orthologous genes was changed after *Pilobolus* and *Mucor* were separated.

Keywords Carbon source · Mucorales · Sodium acetate

Introduction

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC1.2.1.12) is a key enzyme in glycolysis and gluconeogenesis. GAPDH converts glyceraldehyde-3-phosphate into 1,3-bisphosphoglycerate in glycolysis and the reverse reaction in anabolic reaction. Because glucose metabolism is essential for all living organisms, GAPDH is usually expressed constitutively at a higher level. Therefore, the promoter of the *gapdh* gene has been applied to construct an efficient expression vector for fungi (Hirano et al. 1999; Van Bogaert et al. 2008; Wolff and Arnau 2002).

Recent studies revealed that GAPDH is a multifunctional protein (Sirover 2005). In mammalian cells, GAPDH functions in many biological processes such as endocytosis (Robbins et al. 1995), vesicular transport (Tisdale 2001), control of gene expression (Zheng et al. 2003), apoptosis (Ishitani and Chuang 1996), and DNA repair (Krynetski et al. 2001). In several bacteria and yeasts, GAPDH is found in the cell wall. In *Staphylococcus aureus*, cell wall-associated GAPDH is involved in the interaction with host cells and ligands (Pancholi and Fischetti 1997). GAPDHs of *Saccharomyces cerevisiae* (Tdh1, Tdh2, and Tdh3) are distributed not only throughout the cytoplasm but also over the outermost layer of the cell wall, and the cell wall-associated GAPDHs retain their catalytic activity (Delgado et al. 2001). In *Candida albicans*, cell wall-associated GAPDH activity increases in response to starvation and temperature upshift (Gil et al. 2001). *GAP1* of *Kluyveromyces marxianus* is responsible for the flocculent phenotype of yeast (Almeida et al. 2003), but the physiological role is not known for most of the cell wall-associated GAPDHs.

Pilobolus, which belongs to the Mucorales, has been used for many physiological studies such as photomorphogenesis, phototropism, and circadian rhythm (Kubo and Mihara 1986, 1996; Page 1966; Jacob 1961), but no molecular research had been done until recently (Kubo 2009). It is important to develop molecular techniques in *Pilobolus*.

To obtain a homologous promoter that allows efficient recombinant expression in *Pilobolus*, putative GAPDH-encoding genes were cloned from *Pilobolus crystallinus*. Three different *gapdh* genes were identified from

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P. crystallinus and all three genes were expressed. In this article, the structure and the expression pattern of the three putative *gapdh* genes are shown.

Materials and methods

Growing condition

Pilobolus crystallinus (strain NBRC 8561) obtained from the NITE Biological Resource Center, Kazusa, Japan, was used. Sporangiospores were inoculated in MYC medium [1% (w/v) malt extract, 0.2% (w/v) yeast extract, 0.2% (w/v) casamino acids] and cultured at 22°C under continuous white light (7.2 W/m^2). Synthetic medium [0.06% (w/v) ammonium sulfate, 0.05% (w/v) KH_2PO_4, 0.06% (w/v) K_2HPO_4, 0.05% (w/v) $MgSO_4 \cdot 7H_2O$, 0.01% (w/v) NaCl, 0.01% (w/v) $CaCl_2$, 10 mg/l thiamine HCl, 10 mg/l hemin, 1 ml micronutrient solution] (Page 1952, 1960) was supplied with 0.1 M glucose, 0.1 M glycerol, 0.1 M sodium acetate, or 0.1 M sucrose as a carbon source.

Isolation of nucleic acids

Genomic DNA was isolated with a modified version of the CTAB method as reported previously (Kubo 2009). For the isolation of total RNA, mycelium was ground with extraction buffer [4.2 M guanidine thiocyanate, 0.5% (w/v) *N*-lauroylsarcosine sodium salt, 25 mM sodium citrate]. After extraction with phenol:chloroform:isoamyl alcohol (25:24:1) four times, RNA was precipitated with 2.5 volumes 99.5% ethanol and 0.05 volume 5 M NaCl. The pellet was dissolved with RNase-free water, and RNA was precipitated by 2 M LiCl. After ethanol precipitation, the pellet was washed with 70% (v/v) ethanol, dried, resuspended in RNase-free water, and used for reverse transcription-polymerase chain reaction (RT-PCR) (Table 1).

Sequencing

A DYEnamic ET terminator cycle sequencing kit (Amersham Biosciences, Piscataway, NJ, USA) was used for the sequencing reaction. ABI 377 (Applied Biosystems, Foster City, CA, USA) was used for sequencing. Sequence data were analyzed with GENETYX-MAC ver. 9.0 software (Software Development, Tokyo, Japan).

Degenerated PCR

Degenerated oligo primers were designed from conserved amino acid sequences INGFGRIG and WYDNEYGY (Wolff and Arnau 2002). PCR was performed with degenerated oligo primers DGGPDFwd, DGGPDRev to amplify fragments of *gapdh* orthologues in *P. crystallinus*. Oligo primers DGTUBFwd and DGTUBRev were used to amplify a fragment of tubulin cDNA. Amplified fragments were cloned into Bluescript SK$^+$ (Stratagene, La Jolla, CA, USA) and the sequences were determined.

Table 1 Primer sequence used for reverse transcription-polymerase chain reaction (RT-PCR), 5′-RACE, 3′-RACE, and inverse PCR (IPCR)

Primer name	Sequence
DGGPDFwd	5′-ATHAAYGGNTTYGGNMGNATHGG-3′
DGGPDRev	5′-TANCCRTAYTCRTTRTCRTACCA-3′
PCGPD1Fwd	5′-TTATTCCTCTTGATTACATG-3′
PCGPD1Rev	5′-TGTAGCCCAAAATGCCCTTC-3′
PCGPD2Fwd	5′-TTGAATATATGGTCTACATG-3′
PCGPD2Rev	5′-CAGTCAAATCGACTACAGAC-3′
PCGPD3Fwd	5′-ATGGTCTACATGTTCAAGTA-3′
PCGPD3Rev	5′-GGGGATGATGTTGGCACCAG-3′
GPD1-5′Rev1	5′-CATGTAATCAAGAGGAATAA-3′
GPD1-5′Rev2	5′-GGATCGTTAATAGCGACGAC-3′
GPD1-5′Rev3	5′-GGCAGAAATAATGACTTTCTTGGCACCACC-3′
GPD1-3′Fwd1	5′-GAAGGGCATTTTGGGCTACA-3′
GPD1-3′Fwd2	5′-GTGTCCACTGATTTTGTTGG-3′
GPD1-3′Fwd3	5′-GATGCGCCTATGTTCGTTGTAGGTGTCAAT-3′
GPD1-ProFwd	5′-GTTGTTATGATTGAATCTTA-3′
GPD2-5′Rev1	5′-CGTTGAATCTACCATGGACA-3′
GPD2-5′Rev2	5′-CATGTAGACCATATATTCAA-3′
GPD2-5′Rev3	5′-GAAATGGGCAGAGGCACCTTCAGTCTTAGT-3′
GPD2-3′Fwd1	5′-CTCTCTTAACGGAAAGTTGA-3′
GPD2-3′Fwd2	5′-GTCTGTAGTCGATTTGACTG-3′
GPD2-3′Fwd3	5′-GTGGTGTCAACTTGGATGCCTATAAGCCTG-3′
GPD2-ProFwd1	5′-TGGAAGTATGGAACTATATC-3′
GPD2-ProFwd2	5′-TCATTCAGTGTGTACAGAGC-3′
GPD2-ProRev	5′-TGTATAAAGAGTATAAATAT-3′
GPD3-5′Rev1	5′-CTTCAACGGTACCCTTGAAA-3′
GPD3-5′Rev2	5′-TACTTGAACATGTAGACCAT-3′
GPD3-5′Rev3	5′-AAGGTCATCATCTCTGCTCCCTCTGCTGAT-3′
GPD3-3′Fwd1	5′-TGCTGGTGCCAACATCATCC-3′
GPD3-3′Fwd2	5′-AAGCTCACTGGTATGGCCTT-3′
GPD3-3′Fwd3	5′-TCATGCAGTAATATCAATAA-3′
GPD3-3′Fwd4	5′-CAGAGGCCTTGTCAATGGTGGTGAAGACAC-3′
GPD3-ProFwd1	5′-AACCATGGTAATCTCACTCAAG-3′
GPD3-ProFwd2	5′-TACAGGTCAAGATGTGCCC-3′
GPD3-ProFwd3	5′-AACATGATATTACATGCTGA-3′
GPD3-ProRev1	5′-GTTTAATAAGAGAGATGAAT-3′
GPD3-ProRev2	5′-AAATCTCTCTCCTTCTACAC-3′
DGTUBFwd	5′-AAYGTNTWYTWYAAYGARGG-3′
DGTUBRev	5′-TCNSWNGCNGCCATCATRTT-3′
PCTUBFwd	5′-AAAATATGTCCCTAGAGCTG-3′
PCTUBRev	5′-CACTCAGATTACGGTATTGT-3′
RACE primer	5′-AAGACTTCTCTCCGGTTTTTTTTTTTTTTT-3′
Adapter primer	5′-AGCAAGACTTCTCTCCGGTT-3′

RACE, rapid amplification of cDNA ends. N = A, C, G, T; Y = C, T; R = A, G; M = A, C; W = A, T; S = G, C; H = A, C, T

Inverse PCR, 5′-RACE, and 3′-RACE

For inverse PCR (IPR), genomic DNA was digested with *Spe*I or *Xba*I. After circularization with T4 DNA ligase (Promega, Madison, WI, USA), PCR was performed with gene-specific primers GPD1-5′Rev3 and GPD1-3′Fwd3. Nested PCR was performed using GPD1-5′Rev1, GPD1-3′Fwd1, GPD1-5′Rev2, and GPD1-3′Fwd2 for the cloning of the PCGAPDH1 gene. Amplified fragments were sequenced directly or after cloning into Bluescript SK^+ vector. Primers designated as GPD-Pro were used for the sequencing of the promoter region. The primers designated as GPD2 and GPD3 were used for cloning of PCGAPDH2 and PCGAPDH3, respectively.

For 3′-RACE, first-strand cDNA was synthesized by M-MuLV reverse transcriptase [New England Biolabs (NEB), Ipswich, MA, USA] with a rapid amplification of cDNA ends (RACE) primer. PCR was done with a gene-specific primer GPD1-3′Fwd1 and adapter primer, then nested primer GPD1-3′Fwd2 and adapter primer. Amplified fragments were directly sequenced. For 5′-RACE, first-strand cDNA was synthesized by M-MuLV reverse transcriptase with an oligo-$d(T)_{15}$ primer. The 3′-end of the cDNA was polyadenylated with terminal deoxynucleotidyl transferase (Promega). PCR was carried out with a gene-specific primer GPD1-5′Rev1 and RACE primer, then nested primer GPD1-5′Rev2. Amplified fragments were sequenced directly or after cloning into Bluescript SK^+ vector. The primers designated as GPD2 and GPD3 were used for cloning of PCGAPDH2 and PCGAPDH3, respectively.

Semiquantitative RT-PCR

For semiquantitative RT-PCR, first-strand cDNA was synthesized by M-MuLV reverse transcriptase with an oligo-$d(T)_{15}$ primer. The same amount of total RNA was used for template. PCR was performed with the primers PCGPD1Fwd and PCGPD1Rev for PCGAPDH1, PCGPD2Fwd and PCGPD2Rev for PCGAPDH2, PCGPD3-Fwd and PCGPD3Rev for PCGAPDH3, and PCTUBFwd and PCTUBRev for tubulin. The size of amplified DNA for *pcgapdh1*, *pcgapdh2*, and *pcgapdh3* was 723, 621, and 498 bp, respectively. The PCR cycle was 24 for all primers. The PCR product was loaded on an agarose gel, electrophoresed, and stained with ethidium bromide. The density of the band was quantified by a densitometer (Densitograph;

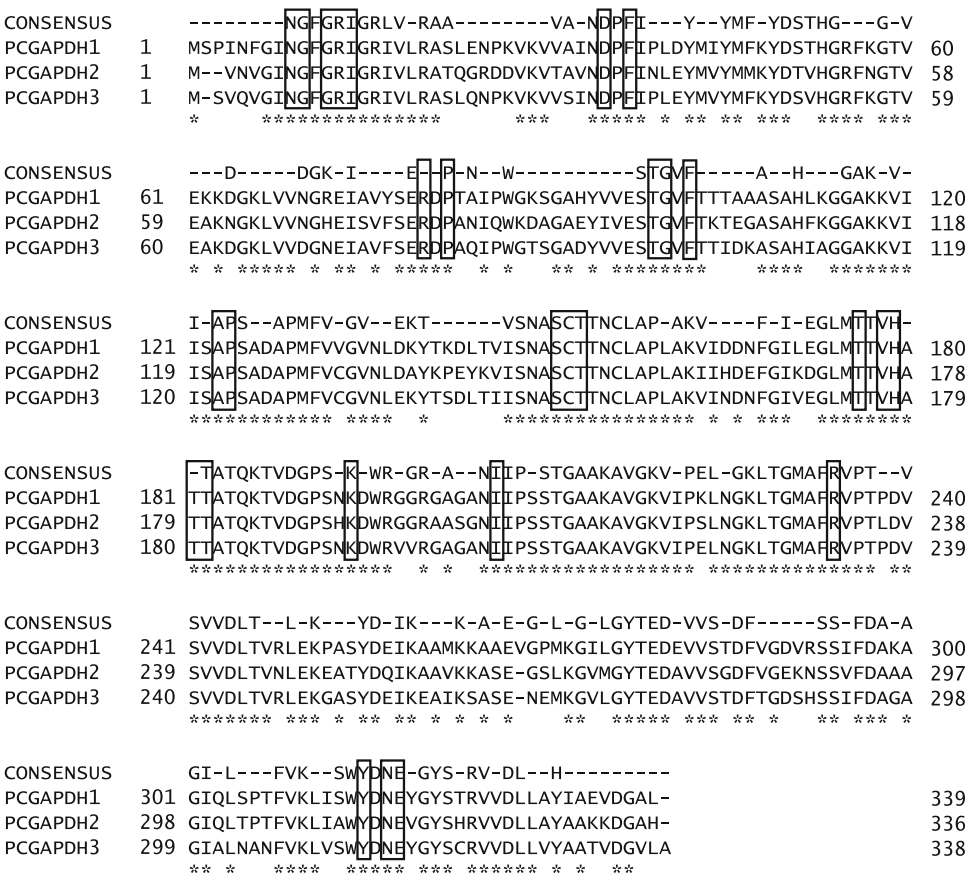

Fig. 1 Deduced amino acid sequences of PCGAPDH1, PCGAPDH2, and PCGAPDH3. Amino acid residues required for catalytic domain, NADH-binding sites, and subunit interaction are *boxed*. *CONSENSUS*, amino acid residues conserved among GAPDH for pig, yeast, and lobster (Harris and Waters 1976). *Amino acid residue conserved between three PCGAPDH proteins

Atto, Tokyo, Japan). Level of expression relative to that of tubulin was shown relative to the level of control. All the steps were done quantitatively.

Phylogenetic analysis

Alignment and phylogenetic analysis of amino acid sequences for GAPDH were performed with T-Coffee (Notredame et al. 2000) and PHYML ver. 3.0 (Guindon and Gascuel 2003), respectively. Amino acids between Asn9 and Glu318 were used for phylogenetic analysis. Bootstrap cycle was 100. Phylogenetic tree was displayed with TREEVIEW ver. 1.6.6 (Page 1996).

Results

Three distinct *gapdh* homologous genes were identified from the PCR products amplified with degenerated primers when genomic DNA as well as cDNA were used as template. These three genes were named *pcgapdh1* (DDBJ/GenBank/EMBL accession no.GQ436781), *pcgapdh2* (GQ436782), and *pcgapdh3* (GQ436783). The nucleotide sequence of full-length cDNA was determined by 5′- and 3′-RACE. The nucleotide sequences showed 71% identity between *pcgapdh1* and *pcgapdh2*, 74% identity between *pcgapdh1* and *pcgapdh3*, and 72% identity between *pcgapdh2* and *pcgapdh3*. *pcgapdh1*, *pcgapdh2*, and *pcgapdh3* were predicted to encode a protein of 339, 336, and 338 amino acids, respectively. The amino acid sequences showed 77% identity between PCGAPDH1 and PCGAPDH2, 84% identity between PCGAPDH1 and PCGAPDH3, and 76% identity between PCGAPDH2 and PCGAPDH3. One extra amino acid (V268) was inserted at 268th of PCGAPDH1. Insertion in the same position is observed only in GPD3 of *M. circinelloides* (Wolff and Arnau 2002). All the three deduced proteins conserved the amino acid residues that are necessary for the catalytic domain (Cys152 and His179 for PCGAPDH1, Cys150 and His177 for PCGAPDH2, Cys151 and His178 for PCGAPDH3), binding sites for NADH, and subunit interaction (Harris and Waters 1976) (Fig. 1).

Figure 2 shows a phylogenetic tree constructed from the amino acid sequences of several fungal GAPDHs. PCGAPDH1, PCGAPDH2, and PCGAPDH3 showed the highest homology with GPD3 (87% identity), GPD1 (83% identity), and GPD2 (87% identity), respectively, of *M. circinelloides*. The amino acid sequence of GAPDH of *Rhizomucor miehei*, another Mucorales fungus, was more similar to those of PCGAPDH1 (83% identity) and PCGAPDH3 (82% identity) than that of PCGAPDH2 (76% identity).

Genomic sequences showed that *pcgapdh1*, *pcgapdh2*, and *pcgapdh3* had three, one, and two introns, respectively. All the introns had consensus 5′ (GTA) and 3′ (PyAG) splice sites for filamentous fungi (Balance 1990). *pcgapdh1* and *pcgapdh3* had an intron in the same position of the C terminal. *pcgapdh2* had no common intron position with the other two *pcgapdh* genes (Fig. 3).

The intron positions were compared with several fungal *gapdh* genes (Fig. 3). The first intron position of *pcgapdh1* was conserved in *gapdh* genes of *R. miehei*, *Aspergillus niger*, and *Agaricus bisporus*. The second intron position was conserved in *gpd2* and *gpd3* of *M. circinelloides*. The

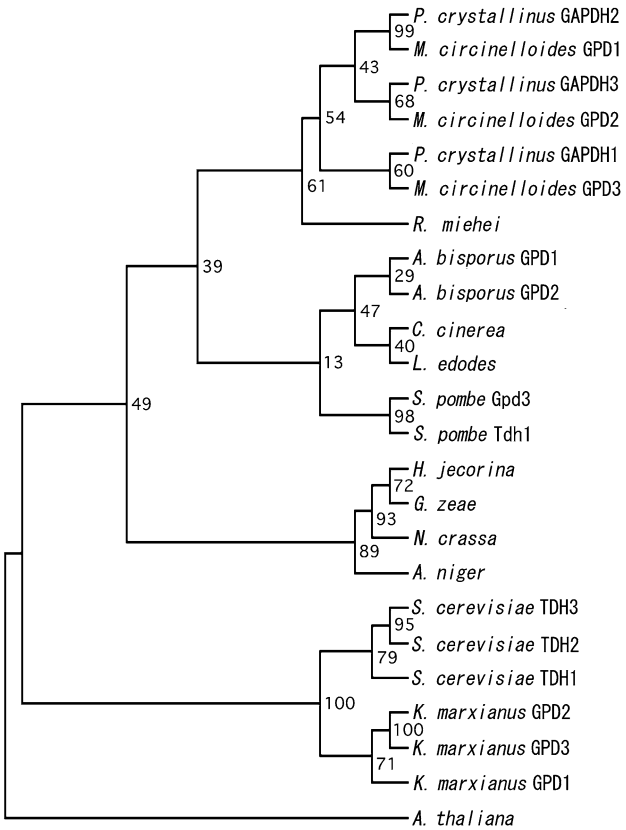

Fig. 2 Phylogenetic tree of GAPDH homologues constructed with the maximum likelihood method. Amino acids between Asn9 and Glu318 were used for constructing the tree. Bootstrap values are shown next to the branching. *Coprinopsis cinerea* (DDBJ/GenBank/EMBL/Swiss-Prot accession no. AB094148.1), *Lentinula edodes* (AB012862.1), *Agaricus bisporus* (M81728.1), *Schizosaccharomyces pombe* Tdh1 (CAA19372), *Schizosaccharomyces pombe* (CAA17812), *Saccharomyces cerevisiae* TDH1 (CAA89343), *Saccharomyces cerevisiae* TDH2 (CAA89531), *Saccharomyces cerevisiae* TDH3 (CAA97218), *Kluyveromyces marxianus* (P84998), *Hypocrea jecorina* (ABK33667), *Gibberella zeae* PH-1(XP_386433.1), *Aspergillus niger* (CAA67966), *Neurospora crassa* (AAB95425), *Mucor circinelloides* GPD1 (AJ293012), *Mucor circinelloides* GPD2 (AJ293013), *Mucor circinelloides* GPD3 (AJ293014), *Pilobolus crystallinus* PCGAPDH1 (GQ436781), *Pilobolus crystallinus* PCGAPDH2 (GQ436782), *Pilobolus crystallinus* PCGAPDH3 (GQ436783), *Rhizomucor miehei* (AF387315), *Arabidopsis thaliana* GAPA (NP_566796)

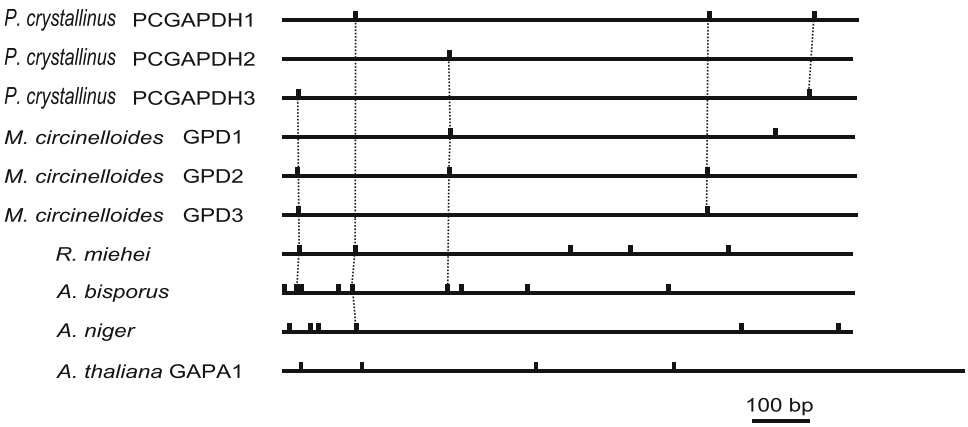

Fig. 3 Intron positions in the coding sequences of several fungal *gapdh* genes. Introns in the same position are linked with *broken lines*. *Bar* indicates 100 bp. *P. crystallinus*, *Pilobolus crystallinus*; *M. circinelloides*, *Mucor circinelloides*; *R. miehei*, *Rhizomucor miehei*; *A. bisporus*, *Agaricus bisporus*; *A. niger*, *Aspergillus niger*; *A. thaliana*, *Arabidopsis thaliana*

position of the intron of *pcgapdh2* was conserved in *gpd1* and *gpd2* of *M. circinelloides* and the *gapdh* gene of *A. bisporus*. The position of the first intron of *pcgapdh3* was conserved in *gpd2* and *gpd3* of *M. circinelloides* and *gapdh* genes of *R. miehei* and *A. bisporus*.

Putative TATA boxes were observed 289 and 333 bp upstream of the start codon of *pcgapdh1*, 94 bp of *pcgapdh2*, and 62 bp of *pcgapdh3*. Putative CAAT motifs were observed 268 and 318 bp upstream of the start codon of *pcgapdh1*, 204 bp of *pcgapdh2*, and 43 bp of *pcgapdh3*. The transcription start point often appears in a CT-rich stretch in filamentous fungi (Gurr et al. 1987). All the three *pcgapdh* genes had the transcription start point in a CT-rich stretch. A putative polyadenylation signal (AATAAA) was observed 36 and 123 bp downstream of the stop codon of *pcgapdh2* and *pcgapdh3*, respectively. The polyadenylation signal was not found in *pcgapdh1* (Fig. 4).

Because the expression of several *gapdh* genes is affected by carbon sources (Fernandes et al. 1995; Wolff and Arnau 2002), I addressed whether the expression of PCGAPDH is affected by carbon sources. Mycelia grown for 2 weeks in MYC medium were transferred to media containing various carbon sources and grown for 4 h. All three *pcgapdh* genes were expressed. Interestingly, the expression of *pcgapdh2* was strongly suppressed when mycelia were grown for 24 h in a medium containing sodium acetate as a carbon source (Figs. 5, 6). The expression of *pcgapdh3* was slightly upregulated by sodium acetate (Fig. 6).

To examine the effect of sugars on the expression of *pcgapdh*s, mycelia grown for 2 weeks in MYC medium were transferred to a synthetic medium without a carbon source and incubated for 1–4 days. The expression of the three *pcgapdh*s was not affected for at least 48 h after removal of sugars (open bars in Fig. 7), except for *pcgapdh3* at 24 h. When the mycelia were transferred to a synthetic medium containing 0.1 M glucose at 0, 24, 48, and 96 h after removal of sugars, no promotive effect on the expression of *pcgapdh*s was observed (shaded bars in Fig. 7). Rather, the expression of *pcgapdh2* was 30–50% reduced by treatment with glucose.

Discussion

In many fungi GAPDH is encoded by a single copy gene (Van Bogaert et al. 2008; Hirano et al. 1999; De Maeseneire et al. 2008), but some fungi have several slightly different *gapdh* genes. *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* have three *gapdh* genes, and these *gapdh* genes are differentially expressed (McAlister and Holland 1985b; Fernandes et al. 1995). On the other hand, transcript of only one of the *gapdh* genes has been detected in other fungi than yeast. *M. circinelloides* has three *gapdh* genes (*gpd1*, *gpd2*, and *gpd3)* but the expression of *gpd2* and *gpd3* is not detected (Wolff and Arnau 2002). *Agaricus bisporus* has two *gapdh* genes (*gpd1* and *gpd2*). They are arranged in tandem in the genome but only the *gpd2* is expressed (Harmsen et al. 1992). Here, I showed that *P. crystallinus* had three *gapdh* genes and that all three genes were expressed. This feature is unique in filamentous fungi.

The function of each *gapdh* gene is not known. All the three putative GAPDH proteins have conserved amino acid residues required for enzymatic reaction, indicating that these three proteins have the possibility of catalyzing the conversion of glyceraldehyde-3-phosphate to 1,3 bisphosphoglycerate. In *S. cerevisiae*, three single mutants (*tdh1*, *tdh2*, and *tdh3*) and two double mutants (*tdh1 tdh2* and *tdh1 tdh3*) can grow using glucose as carbon source but the *tdh2 tdh3* double mutant cannot (McAlister and Holland 1985a), indicating a redundant role of TDH2 and TDH3 in glucose metabolism.

Otherwise, some of the PCGAPDH might have a different function from glucose metabolism. It is shown that GAPDH is multifunctional in various cellular and

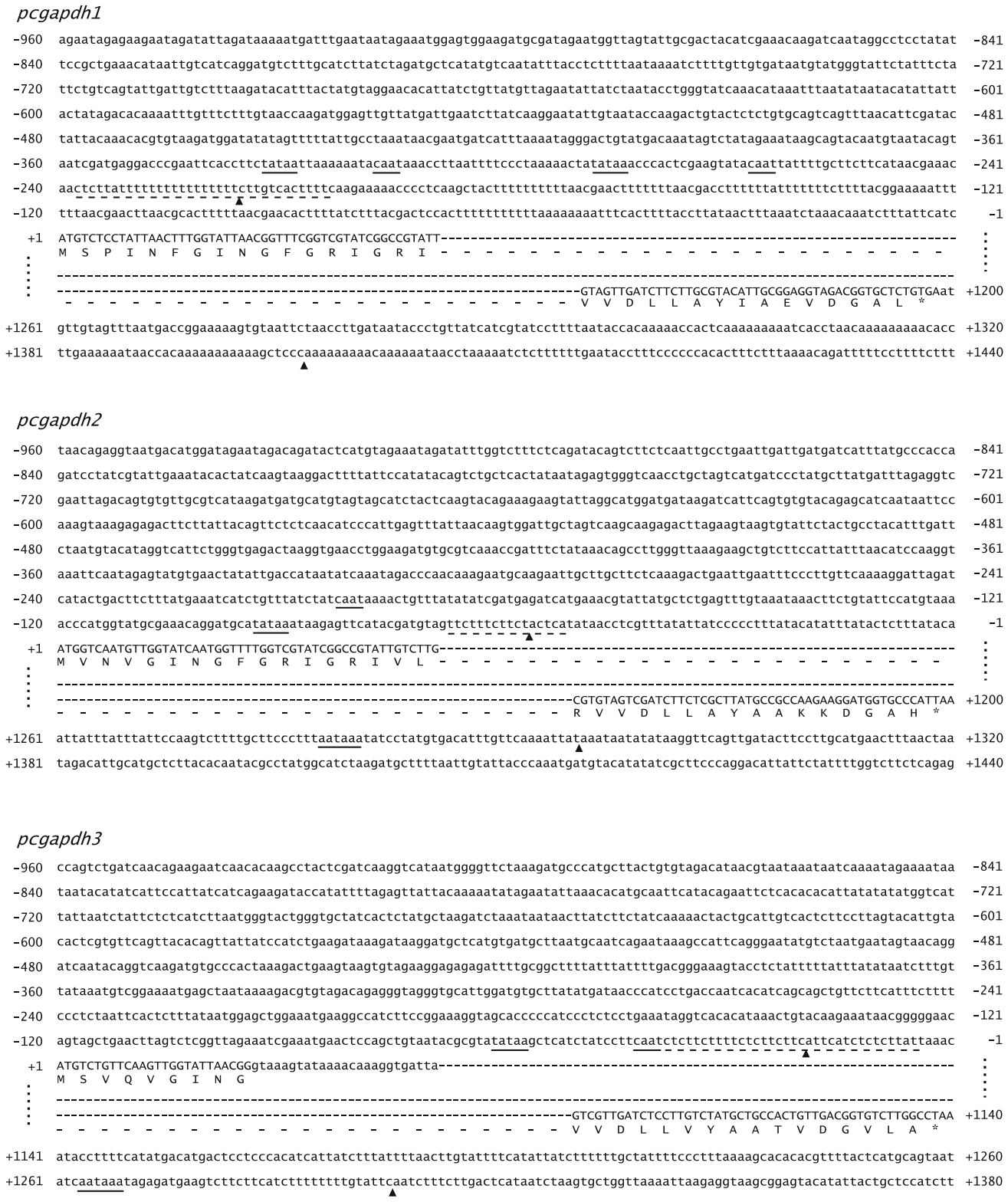

Fig. 4 The sequences of the 5′- and 3′-flanking regions of *pcgapdh1*, *pcgapdh2*, and *pcgapdh3*. The coding regions are indicated in *uppercase letters* and the predicted amino acid sequences for the regions are given in the *one-letter code*. The *middle part* of the coding region is omitted. Putative transcription start site and termination site are shown by *arrowheads*. TATAA, CAAT, and AATAAA motifs are *underlined*. The CT-rich stretch is underlined with a *broken line*. *Numbers* shows the nucleotide position from the translational start site

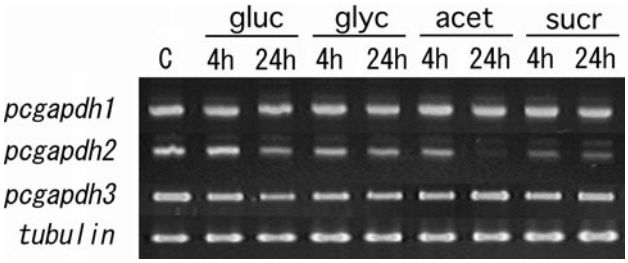

Fig. 5 Effect of several carbon sources on the expression of *pcgapdh1*, *pcgapdh2*, and *pcgapdh3*. Mycelia grown in liquid MYC medium for 14 days (*C*) were transferred to a synthetic media containing 0.1 M glucose (*gluc*), 0.1 M glycerol (*glyc*), 0.1 M sodium acetate (*acet*), or 0.1 M sucrose (*sucr*) as a carbon source. After 4 and 24 h, RNA was extracted and the expression of *pcgapdh* genes was examined by semiquantitative RT-PCR. PCR cycles were 24 for all primers. Tubulin was used as the control

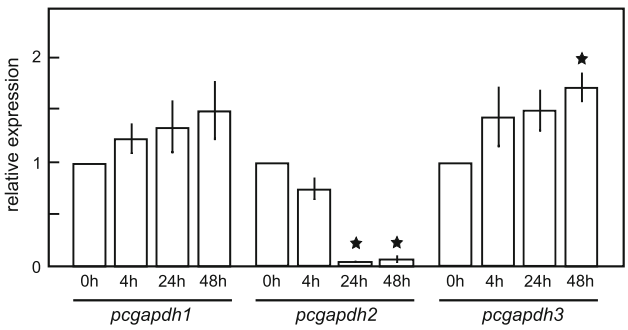

Fig. 6 Effect of sodium acetate on the expression of *pcgapdh* genes. Mycelia grown in liquid MYC medium for 14 days were transferred to a synthetic media containing 0.1 M sodium acetate as a carbon source. RNA was extracted 0, 4, 24, or 48 h afterward, and the expression of *pcgapdh* genes was examined by semiquantitative RT-PCR. Levels of expression relative to that of tubulin are shown relative to the level without sodium acetate treatment (0 h). PCR cycles were 24 for all primers. Each value is the average and SE from 7 PCR reactions. *Asterisks* indicate significant differences from the control (0 h) ($P < 0.05$)

molecular processes in mammals (Sirover 2005). Some fungal GAPDHs also show non-glycolytic function. In *Candida albicans*, GAPDH is found in the cell wall and its activity is controlled by nutrient condition and temperature (Gil et al. 2001). In *K. marxianus*, GAP1 accumulates in the cell wall and overexpression of GAP1 induces aggregation of cells, showing the flocculent phenotype (Almeida et al. 2003).

Because of their essential role for all living organisms, most GAPDHs are constitutively expressed. However, several GAPDHs are regulated especially when GAPDH is encoded by multiple genes. In *S. cerevisiae*, TDH1 protein is only accumulated when cells enter the stationary phase or in heat-shocked cells, whereas accumulation of TDH2 protein is repressed by heat shock (Boucherié et al. 1995). Expression of some GAPDH is controlled by the carbon source. In *K. marxianus*, GAP1 and GAP2 are expressed in the presence of glucose. The expression of GAP2 is

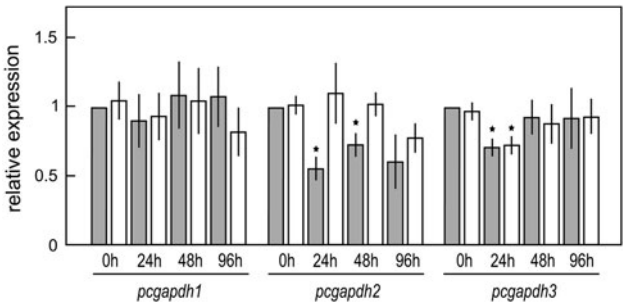

Fig. 7 Effect of glucose to starved mycelia. Mycelia grown in liquid MYC medium for 14 days were grown in a synthetic media without carbon source for 0–96 h, then transferred to a synthetic media containing 0.1 M glucose. After 4 h, RNA was extracted, and the expression of *pcgapdh* genes was examined by semiquantitative RT-PCR. PCR cycles were 24 for all primers. Levels of expression relative to that of tubulin are shown relative to the level of control (+glucose, 0 h). *Shaded bars*, +glucose; *open bars*, −glucose. Each value is the average and SE from 5 PCR reactions. *Asterisks* indicate significant differences from the control ($P < 0.05$)

strongly reduced in a medium supplemented with ethanol as a carbon source, but the expression of GAP1 is not affected (Fernandes et al. 1995). The expression of GPD1 of *M. circinelloides* is promoted by glucose (Wolff and Arnau 2002). Here, I show that the expression of *pcgapdh2* was suppressed by sodium acetate and slightly suppressed by glucose. It is probable that acetate induces feedback suppression because acetate is a downstream metabolite of glycolysis. However, it is curious that glucose also suppresses the expression of *pcgapdh2*. PCGAPDH2 may have another function than glycolysis. Otherwise, acetate and glucose might suppress the expression of *pcgapdh2* by inhibiting the formation of the trophocyst, in which *pcgapdh2* is specifically expressed (unpublished data).

The phylogenetic tree showed that PCGAPDH1, PCGAPDH2, and PCGAPDH3 were counterparts of GPD3, GPD1, and GPD2, respectively, of *M. circinelloides.* Apparently the three GAPDHs had diverged before *Pilobolus* and *Mucor* separated. The expression pattern, however, was different between these orthologues. *pcgapdh2* was downregulated by glucose and sodium acetate, but its counterpart *gpd1* is upregulated by glucose (Wolff and Arnau 2002). *pcgapdh1* and *pcgapdh3* showed high expression but *gpd2* and *gpd3* show no expression (Wolff and Arnau 2002). The role of these orthologues seems to have been changed after *Pilobolus* and *Mucor* separated.

Besides the importance of its function, GAPDH is intensively examined to construct an efficient expression vector for fungi because GAPDH is usually expressed constitutively at a higher level (Hirano et al. 1999; Van Bogaert et al. 2008; Wolff and Arnau 2002). Because *pcgapdh1* and *pcgapdh3* were expressed constitutively at a high level, their promoters can be good tools for an expression vector of *Pilobolus*.

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